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EVALUATION OF TRIETHYLAMMONIUM PHOSPHATE AND FORMATE–ACETONITRILE MIXTURES AS ELUENTS FOR HIGH-PERFORMANCE GEL PERMEATION CHROMATOGRAPHY*

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SUMMARY

Trialkylammonium phosphate or formate and more specifically triethylammonium phosphate or formate buffers (pH < 3) in the presence of 15–30% acetonitrile have been shown to be compatible with the protein analysis column PAC I-125 of Waters Associates for gel permeation of peptides and proteins. A linear relationship between log molecular weight (1,000–44,000) *versus* retention time is obtained. The effect of salt and organic modifier concentrations as well as temperature and column load was studied using a mix of proteins and peptides of varying isoelectric points as well as hydrophobicity. The advantages of that system (beside being already widely used in reversed-phase high-performance liquid chromatography) include low ionic strength, UV transparence and compatibility (after elimination of the volatile acetonitrile) with most biological systems. Applications include purification of iodinated trace and natural products and precise molecular weight determination.

INTRODUCTION

Fractionation of proteins according to size utilizing cross-linked dextran or polyacrylamide gel columns was first demonstrated by Porath and Flodin[1] in 1959. This technique has become the most widely accepted method for separation and molecular-weight determination of hydrophilic as well as some hydrophobic macromolecules using aqueous buffers with or without organic modifier. While this technique might not be unique in its ability to resolve and separate proteins (*i.e.*, ion-exchange and partition chromatographies or electrophoresis on these gels are widely used) it certainly is simple and effective.

With the development of new supports which are not compressible (in contradistinction with the soft gels mentioned earlier) the basic principle of this technique

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has now been applied under higher pressures and has been given the acronym of HP-GPC for high-performance gel permeation chromatography. One major obstacle to be overcome was to find non-compressible supports compatible with the biomaterials to be chromatographed *i.e.*, (a) non-specific adsorption had to be minimized and (b) controlled pore size had to be achieved. This early work has been reviewed by Cooper and Van Derveer[2].

More recently, Hashimoto *et al.*[3] have described protein separations on chemically modified silica gel base supports, TSK-Gel PW-type columns whereas Fukano *et al.*[4] and later Rokushika *et al.*[5] have used TSK-Gel 2000 and 3000 SW columns to respectively demonstrate the usefulness of such columns for the separation and recovery of biologically active proteins and enzymes as well as the separation of saccharides and water-soluble synthetic polymers. The influence of flow-rate on plate height and retention volumes for these two sets of columns was also investigated. These investigators found a linear relationship between the logarithm of the molecular weight *versus* K (distribution coefficient) for proteins of molecular weights ranging from ferritin (mol.wt. 480,000) to insulin (mol.wt. 6000) using the TSK 3000 SW column and a phosphate buffer (0.01 M phosphate pH 6.5 containing 0.2 M sodium sulfate).

It is of interest that all these studies as well as those recently presented at the *LC Symposium III, October 1979, Boston*[6–11] agree with the fact that without addition of a detergent, the separation of proteins according to size is only possible by adjusting the pH and the ionic strength of the eluent. Consequently, relatively high concentrations of non-volatile buffers must be employed to increase ionic strength which tends to minimize electrostatic (ionic) interactions of positively charged proteins ($pI > 8$) with the negatively charged surface[9,12] resulting in a reduced elution volume. At the same time it will also decrease electrostatic repulsion of negatively charged proteins with the negative surface and result —for those proteins— in a larger elution volume than theoretically predicted. Schmidt *et al.*[12] went one step further and, using a diol phase (LiChrosorb Diol from E. Merck, Darmstadt, G.F.R.) found that at pH 5.0 this phase showed residual ionic charges below an ionic strength of 0.2 M; however, while the protein-stationary phase ionic interactions were neutralized above μ 0.20 M, unusually hydrophobic proteins (especially lysozyme and chymotrypsinogen) started to exhibit hydrophobic interactions with the support. Similar decreases in ionic interaction of proteins with Sepharose B[13] and Sephacryl S-200[14] when $\mu \geqslant 0.2$ M have been suggested. In practice low ionic strength generally results in poor resolution and recovery of proteins having widely different isoelectric points or hydrophobic character. Another approach reported by Kato *et al.*[15] using TSK-Gel SW type columns was the inclusion of sodium dodecyl sulfate in their mobile phase (ion pairing and/or detergent effect). Resolution again was highly dependent upon salt concentration (sodium sulfate: ideally 0.05–0.2 M).

In order to circumvent the problem of not being able to use successfully volatile buffers such as ammonium acetate and bicarbonate (10 mM) Waterfield and Scarce[6] chose to succinylate or citraconylate their proteins to improve elution characteristics on protein analysis columns (PAC) I-125 columns (Waters Assoc., Milford, MA, U.S.A.) as well as allow for monitoring at 254 nm.

None of these solutions to apparently basic problems (*i.e.*, those inherent to the support as well as those inherent to the proteins *i.e.*: solubility at different pH and

ionic strength) satisfied our need for a UV transparent biologically compatible and if possible voltile buffer, which would allow for good resolution and recovery of a wide range (in terms of their ionic and hydrophobic character) of proteins and peptides. We believe that the solvent system described here may have most of the ideal characteristics mentioned earlier. It represents an expansion of our work done with the triethylammonium formate (TEAF) and triethylammonium phosphate (TEAP) buffers which have proven so effective as solvents for reversed-phase high-performance liquid chromatography[16] (RP-HPLC).

The TEAP and TEAF buffers in the presence of a certain amount of acetonitrile are shown to be compatible with the PAC I-125, and to give good resolution and good recoveries for the peptides and proteins studied. Linearity of dose/response as well as calibration curve for molecules ranging from acetic acid (mol.wt. 60) or thyrotropin releasing factor (mol.wt. 363) to globulins (mol.wt. 150,000) —including proteins with quite different isoelectric points— , are presented. Concentration of the organic modifier and of the salt in the buffer is defined, and the temperature effects on resolution investigated.

MATERIALS AND METHODS

Apparatus

The apparatus consists of Waters Assoc. Models: 204 liquid chromatograph, U6K injector, two 6000A pumps, 660 programmer, Model 450 multiwave-length UV/visible detector, Infotronics Model 110 integrator, and Linear Instruments Model 445 chart recorder. Two PAC I-125 columns Nos. 093593 and 093594 were used in these studies. Full-scale absorbance is expressed in a.u.f.s. The data were analyzed with a Hewlett-Packard Model No. 9830A computer using a least-squares analysis program and graphed using Model No. 9866A printer.

Composition of the TEAP and TEAF buffer[16]

The TEAP buffer was obtained by bringing the pH of 0.25 *N* phosphoric acid to 2.25 with redistilled (over *p*-toluenesulfonylchloride) triethylamine. Several liters were made at one time which were filtered over a C_{18} cartridge in Waters Assoc. Prep LC-500. As a result, any hydrophobic UV-absorbing material was eliminated, thus allowing for clean washes at the end of gradients in our analytical system. The bulk of the buffer was kept in a cold room since bacterial contamination was observed during summer months. Prior to use, aliquots are being degassed and stirred under house vacuum for 5 min.

The TEAF buffer was obtained by bringing the pH of 0.25 *N* formic acid to 3.0 with redistilled triethylamine. It was millipore filtered before use to eliminate any solid particles that might plug the columns. For studies involving lower concentrations of TEAP buffer, simple dilutions were made from the original stock.

Conditions

The A buffer was pure or diluted TEAP whereas the B buffer was a mixture of 40% A and 60% acetonitrile. Using these two solutions both pumps delivered 0.5 ml/min for a final concentration of 30% acetonitrile.

All experiments were run at 50°C unless otherwise indicated. Conditions used

in each experiment are described in the legends of each table or figure. High temperature was controlled within 1°C by immersing the columns in a thermoregulated water bath. Flow-rate was 1.0 ml/min; chart speed was 1 cm/min; column back pressure was 100–150 p.s.i.

RESULTS AND DISCUSSION

It was shown by several groups[16–18] that peptides and even small proteins (mol.wt. < 12,000) could be eluted from reversed-phase supports using appropriate conditions (*e.g.* buffer composition and pH, organic modifier, column support, temperature, flow-rate and gradient shape have been optimized).

The reasons that larger proteins (mol.wt. > 12,000) could not be eluted include insolubility of the proteins under the chromatographic conditions, inadequate pore size of the support, and inappropriate kinetics of exchange of the proteins between the different phases.

With the availability of non-compressible hydrophilic supports (PAC I-125 in this case) which had been designed for the chromatography of large hydrophilic polymers/proteins and using our past experience in peptide/protein separation using HPLC technology, we undertook to test the compatibility of TEAP/TEAF buffers on such a column for peptide/protein separation and/or molecular-weight determination while keeping in mind our ultimate goal of developing a UV transparent, biologically compatible or volatile buffer which would give high resolution and good recoveries.

Fig. 1 shows the effect of acetonitrile concentration on the resolution of different peptides and proteins (see conditions in the legend). As could have been predicted, the separation of larger proteins is improved at lower concentration of acetonitrile (better overall solubility) whereas for smaller peptides, higher concentration of acetonitrile seem favorable. However, as is often the case for peptides and proteins exhibiting a large spectrum of solubility characteristics due to their inherent primary and tertiary structures (low to high isoelectric points, more or less hydrophobic and globular or random in conformation), no generalization is possible. For the first time however a combination of an aqueous buffer and a significant amount of an organic modifier is being successfully used for the elution of a peptide/protein mixture using a high-pressure system (see ref. 12 for effect of added ethylene glycol to mobile phase).

In the range of 15–30% acetonitrile the different components of the mixture are being separated according to size in a gel permeation mode with very little non-specific adsorption ($V_t/V_o \leqslant 1.1$, < 1.3 calculated by Regnier *et al.*[19]; (V_t = internal volume of column = $V_t - V_o$; V_t = elution volume of the excluded bovine serum albumin (BSA)). Good peak symmetry is another indication of non-specific adsorption. It is noteworthy that Sokolowski and Wahlund[20], studying peak tailing and retention behavior of tricyclic antidepressant amines and related ammonium compounds by RP-HPLC, confirmed our earlier results[16] showing that addition of alkylammonium ions to the mobile phase reduced tailing. Their extensive studies indicated that the nature and the concentration of the added alkylamines as well as the nature of the column support were critical for good peak symmetry or selectivity, respectively. Whereas triethylamine had an acceptable asymetric factor value of two, dimethyloctylamine for example, had one close to one (excellent). Whether one of

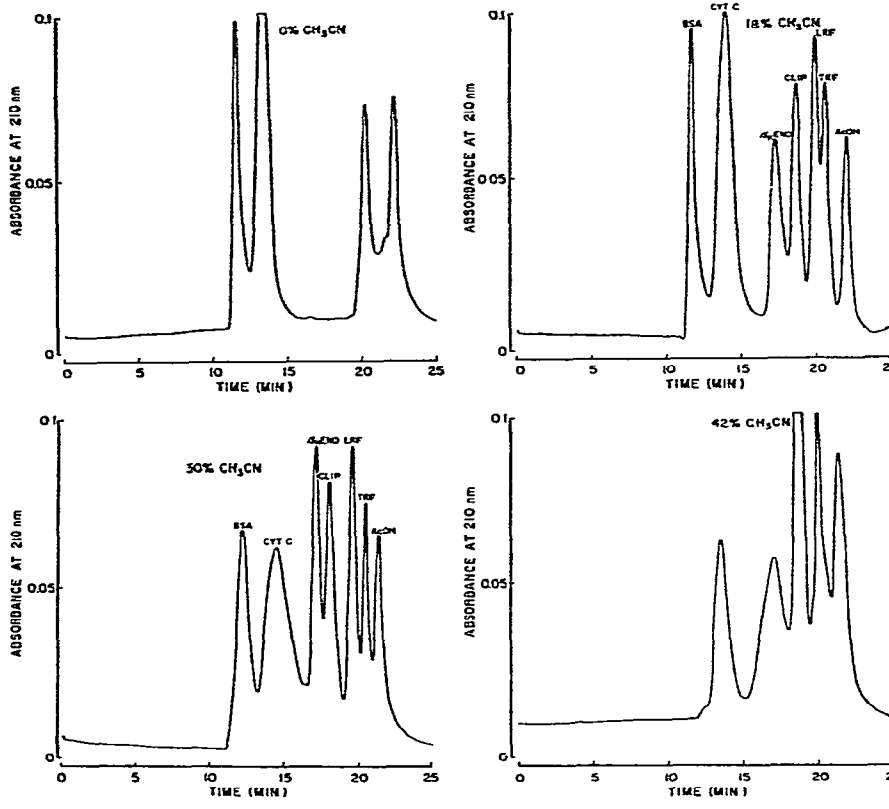

Fig. 1. Influence of acetonitrile concentration on elution pattern. Conditions: load, 50 μl protein–peptide mixture; solvent system, TEAP pH 2.25–acetonitrile as shown. Retention times (sec) for the different components are for 18% and 30% acetonitrile respectively: BSA, 695 and 729; cyt *c*, 829 and 861; β-endorphin (human), 1027 and 1011; CLIP, 1111 and 1075; LRF, 1185 and 1167; TRF, 1227 and 1221; acetic acid, 1311 and 1273.

those amines or other organic modifiers such as methanol, *n*-propanol, isopropanol, *n*-butanol, tetrahydrofuran and pyridine currently used with success in RP-HPLC of peptides will be also compatible with HP-GPC of peptides and proteins remains to be established.

Also noteworthy is the low pH (below most isoelectric points) of the aqueous buffer used for the separation shown in Fig. 1. A low pH (< 3) is recommended in RP-HPLC for most peptides[16–18] (exception: acidic peptides which are insoluble under those conditions: for example, gastrin I for which a dilute 1:1 TEAP buffer at pH $\approx$ 6.5 is recommended[21]). Obviously, proteins which would be insoluble under the conditions used, could hardly be expected to elute from any column. It is remarkable however that among the proteins present in this mix, cytochrome *c* has a p*I* of 10.6 (ref. 22) whereas BSA has a p*I* of 4.4–4.8 (ref. 23). Recovery studies using integrated areas under the peaks and different loads (5, 10, 20 and 40 μg) have shown good linearity for all components of the mixture when using 30% acetonitrile and the TEAP pH 2.25 buffer at room temperature. In that experiment, retention times

remained constant, indicating that overloading had not occurred. Day-to-day reproducibility appeared good (variability $< 1\%$ in retention times) but was not extensively studied since it is very dependent on pump performance.

Fig. 2 shows a separation similar to that reported in Fig. 1. Except for a different composition of the peptide/protein mix (see legend) which includes indole (a hydrophobic small substance: mol.wt. 117), the only variable is the concentration of the TEAP buffer in the eluting solvent. These separations are to be compared with that shown in Fig. 1 (30% acetonitrile); even though no dramatic change in resolution is observed, dilution of the TEAP buffer to 0.0625 *N* or 0.02 *M* (Fig. 2b) may give better results.

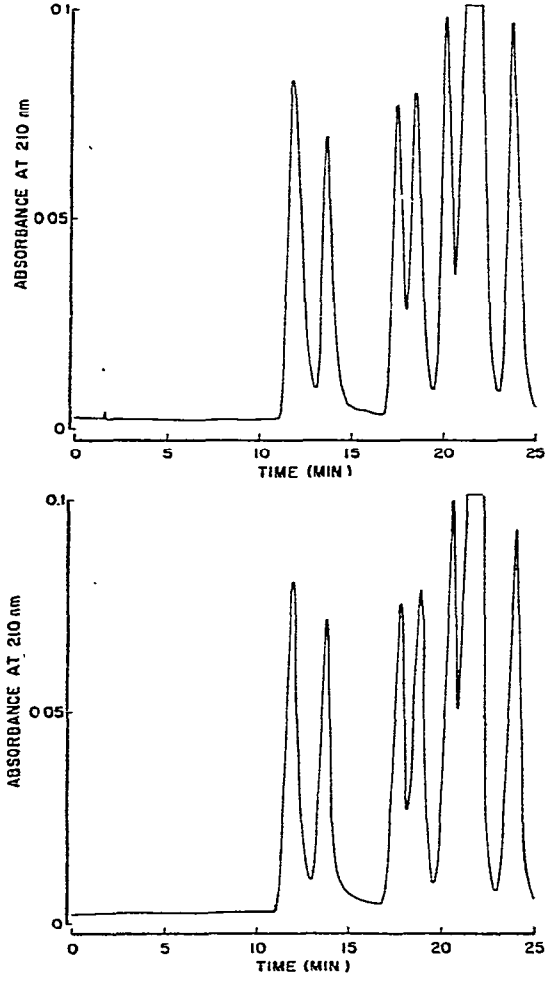

Fig. 2. Influence of TEAP concentration on elution pattern. Conditions: load, 35 μl protein–peptide mixture; solvent system top, TEAP pH 2.25–water (1:1)–30% acetonitrile; bottom, TEAP pH 2.25–water (1:3)–30% acetonitrile. Retention times (sec) for the different components are for TEAP–water (1:1) and (1:3) respectively: BSA, 710 and 700; cyt *c*, 818 and 810; β-endorphin (human), 1046 and 1048; CLIP, 1108 and 1112; LRF, 1206 and 1214; TRF + TEAF, 1300 and 1286; indole, 1424 and 1420.

TABLE I

EFFECT OF TEAP CONCENTRATION ON RETENTION TIMES OF PEPTIDES AND PROTEINS

Conditions: aqueous buffer–acetonitrile (7:3) isocratic. Clip = adrenocorticotropic hormone (18–39).

Protein	*Retention times (sec)*		
	TEAP 2.25	*TEAP 2.25–water (1:1)*	*TEAP 2.25–water (1:3)*
BSA	729	710	700
Cytochrome *c*	861	818	810
β_h-Endorphin	1019	1046	1048
Clip	1075	1108	1112
LRF	1167	1206	1214

Table I shows more accurately the effect of the TEAP concentration on retention times of peptides and proteins. Whereas proteins elute earlier at low concentration of the buffer than at higher buffer concentration, peptides have the opposite tendency. No simple interpretation of those results can be given: two phenomena may be involved: (a) a dependence of V_i upon TEAP concentration would be compatible with some interaction of the eluent and the stationary phase[20]; (b) the particular solvent system has an effect on the Stokes' radius of the peptide and protein studied. At high concentration of TEAP, proteins appear smaller than they really should be (salting out effect) whereas peptides appear larger than they really are (ion pairing and solvation effect).

The fact that indole is being unexpectedly retarded in this system —even though V_i/V_o is still < 1.3— may indicate that other compounds may also show anomalous behavior in this system (for example: very hydrophobic luteinizing hormone releasing factor (LRF) antagonist; cyclic peptides such as insulin and LRF analogs; see Fig. 4, Tables II and III.

We then investigated the effect of temperature on the standard peptide/protein mix. Fig. 3 (see legend for conditions) clearly shows the advantage of working at higher temperatures.

Using the retention times obtained from Table II (dilute TEAP 1:1), we plotted log molecular weight *versus* retention times (in sec). Fig. 4 shows a linear relationship for molecular weights ranging from 1000 to 44,000. This is somewhat different from the suppliers specifications (mol.wt. range 2000–80,000) but must be accounted for by the unusual composition of the eluting buffer. The correlation coefficient derived from linear regression analysis was found to be —0.985. BSA and in another similar experiment, human γ-globulins were excluded whereas TRF, [Met^5]-enkephalin, LRF$^{5-10}$, acetic acid, and indole also fell outside of the linear range. Insulin which was not reduced eluted with an apparent low molecular weight as expected. It is interesting to note that at this pH, peptides and proteins with a high p*I*: bradykinin, dynorphin, cytochrome *c* and soybean trypsin inhibitor appear to be larger in size than they are, probably due to some ion pairing effect or repulsive effect of the support, both already discussed.

Fig. 5 (see legend for exact conditions) shows a similar separation using the TEAF buffer, thus demonstrating that similar resolution can be achieved with a volatile buffer. It was shown that this buffer has a different selectivity under reversed-

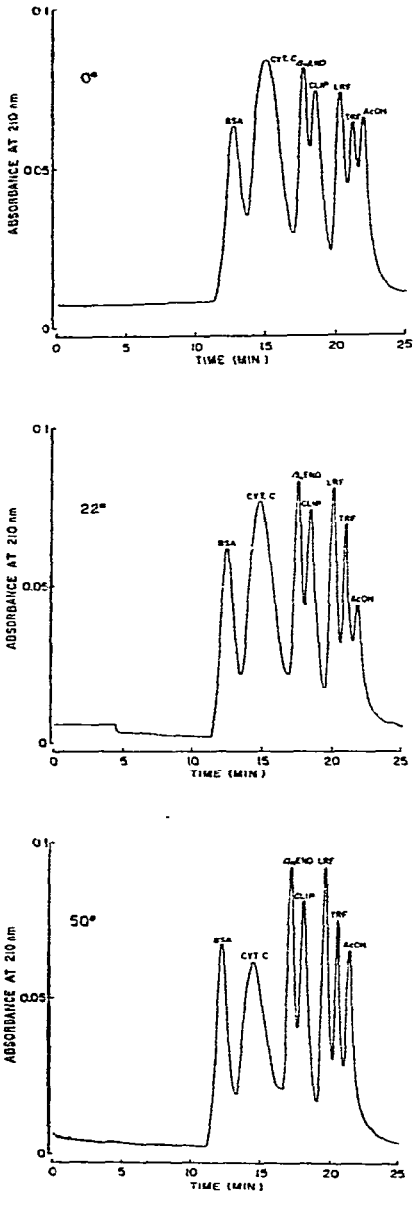

Fig. 3. Influence of temperature on elution pattern. Conditions: load; 50 μl protein–peptide mixture; solvent system TEAP pH 2.25 + 30% acetonitrile. Temperature as shown. Retention times (sec) for the different components are for 0°, 22° and 50°C respectively: BSA, 766, 740, 729; cyt *c*, 900, 878, 861; β-endorphin (human), 1060, 1042, 1011; CLIP, 1112, 1096, 1075; LRF, 1216, 1192, 1167; TRF, 1276, 1246, 1221; acetic acid, 1314, 1298, 1273.

phase conditions[29] and we do not want to exclude at this stage the possibility that it might also be the case in HP-GPC for certain compounds. Whereas 0.1% TFA, for example, is a very good solvent in RP-HPLC, it is interesting, that it has been found inappropriate with and without acetonitrile for the HP-GPC of our peptide mix.

TABLE II

PEPTIDES AND PROTEINS USED IN THIS STUDY

Retention times are averages of 3–4 runs done on different days. Values of acetic acid, indole, TRF, [Met^5]-enkaphalin, LRF (5–10), insulin and γ globulins are not introduced in calibration curve. □, values not introduced in the calibration curve; ⁎, values used for the calibration curve.

Peptides	*Reference*	*Nos. of residues*	*Molecular weight*	*Retention time (sec)*
1□ Acetic acid			60	1370 ± 9
2□ Indole			117	1449 ± 1
3□ Thyrotropin releasing factor (TRF)*		3	363	1365 ± 2
4□ [Met^5]-enkephalin		5	573	1321 ± 5
5□ LRF (5–10) Ac-Tyr–Gly–Leu–Arg–Pro–Gly-NH_2*			718	1296 ± 3
6⁎ Oxytocin*		9	1008	1302 ± 5
7⁎ Bradykinin*		9	1059	1269 ± 3
8⁎ Luteinizing hormone releasing factor (LRF)*		10	1182	1278 ± 3
9⁎ LRF antagonist**		10	1396	1318 ± 2
10⁎ Substance P*		11	1346	1243 ± 2
11⁎ Dynorphin*	24	13	1602	1203 ± 5
12⁎ α-Melanocyte stimulating hormone*		13	1663	1238 ± 4
13⁎ Neurotensin*		13	1672	1221 ± 3
14⁎ Bombesin*		14	1619	1250 ± 2
15⁎ Somatostatin*		14	1638	1266 ± 7
16⁎ CLIP (human)*		22	2463	1172 ± 2
17⁎ β-Melanocyte stimulating hormone*		22	2658	1198 ± 4
18⁎ ACTH (1–24)*		24	2930	1166 ± 8
19⁎ Gastrin releasing peptide*	25	27	2785	1147 ± 2
20⁎ Somatostatin (1–28)*	26	28	3137	1164 ± 5
21⁎ Vasoactive intestinal peptide (VIP)**		28	3322	1148 ± 10
22⁎ Glucagon**		29	3479	1155 ± 7
23⁎ β-Endorphin (human)*		31	3461	1118 ± 4
24□ Insulin**		51	5700	1116 ± 3
25⁎ Cytochrome *c**			≈12,200	898 ± 8
26⁎ Trypsin inhibitor (soybean)**			≈14,300	857 ± 3
27⁎ Growth hormone (human)*			≈22,000	896 ± 2
28⁎ Trypsin (bovine)**			≈23,000	897 ± 1
29⁎ Chymotrypsinogen (bovine)**			≈25,500	869 ± 9
30⁎ Carbonic anhydrase**			≈29,500	775 ± 2
31⁎ Albumin (egg)**			≈44,000	753 ± 4
32□ γ Globulins**			≈150,000	732 ± 5

* These peptides were synthesized in our laboratory using solid phase methodology[27].

** LRF antagonist: [Ac-dehydro^{3,4} L-Pro^1, *p*Cl-D-Phe^2, -D-Trp^{3,6}, N$^{\alpha}$MeLeu^7]-LRF.

VIP was a gift from Dr. S. Lavielle; glucagon, Lot 258-V016-235, gift from Eli Lilly; insulin, Lot 615-D63, gift from Eli Lilly; cytochrome *c*, Sigma (St. Louis, MO, U.S.A.) Lot 106C-7300, p*I* 10.6 (ref. 22); trypsin inhibitor, Sigma Lot Σ 51c-8130; growth hormone (human), Lot 9300^8, gift from Calbiochem; trypsine (bovine), Sigma Lot Σ 106c-8105, p*I* 10.8 (ref. 28); chymotrypsinogen (bovine), Sigma Lot A-124c-8200, p*I* 9.5 (ref. 22); carbonic anhydrase, Sigma Lot Σ 43c-8410; albumin (egg), Sigma Lot Σ 123c-8110, p*I* 4.7 (ref. 22); γ globulins, Miles Labs. (Slough, Great Britain) Lot 82-455-1, serum albumin (bovine) used in these studies was also from Miles, Lot 81-100-2 39, p*I* = 4.4–4.8 (ref. 23).

Applications

This system is being tested in our laboratory for the purification of iodinated traces of larger hormones (growth hormone, thyroid stimulating hormone) to be used in radioimmunoassays. We have also used it to determine the molecular weight of

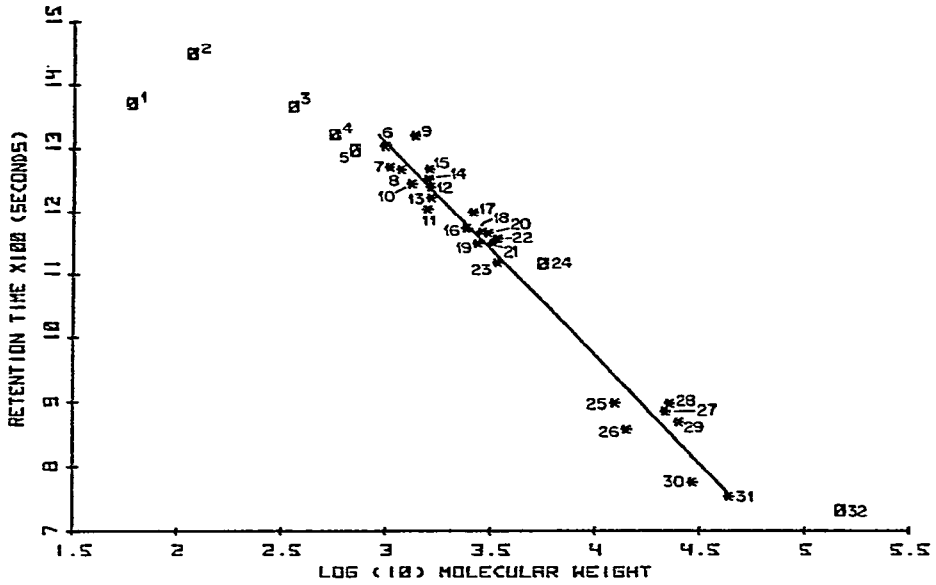

Fig. 4. Correlation of retention time and log mol.wt. in TEAP pH 2.25–water (1:1)–30% acetonitrile at 50°C. See Table II for compound identification.

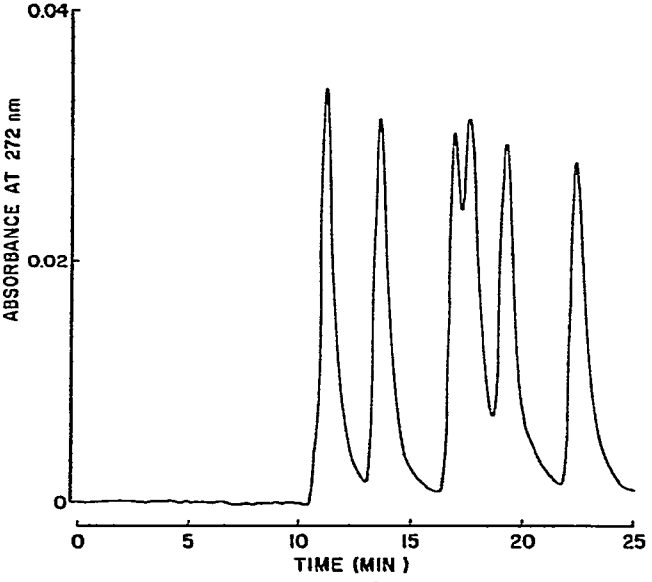

Fig. 5. Separation using TEAF–acetonitrile. Conditions: load, 25 μl protein–peptide mixture; solvent system, TEAF pH 3.0–30% acetonitrile. Retention times (sec) for the different components are BSA, 678; cyt *c*, 822; β-endorphin (human), 1026; CLIP, 1064; LRF, 1162; Indole, 1350.

several unknown biologically active molecules now being purified (ex. mammalian bombesin-like substance).

Advantages over other existing methods to determine molecular weights include high sensitivity, accuracy as well as rapidity of the method. Accurate retention

times and integrated areas measured at 210 nm allow for more precision than could be obtained routinely on soft gel columns. We have indeed been able to show reproducible and statistically significant differences in retention times for four analogous peptides of molecular weights of the order of 1350, two of which are linear, while the other two are cyclized through the backbone (Table III). The linear peptides have a molecular weight of 1367.5 and have charged N-termini at pH 2.25 whereas the cyclic peptides have a molecular weight of 1349.5 (18 less than the linear peptides) and have no end group charge. The cyclic peptide that had a smaller Stoke's radius and were uncharged, eluted later than the linear ones (see Table III).

TABLE III

Conditions: each peptide (10 μg) was eluted successively; buffer, TEAP pH 2.25–30% acetonitrile; absorbance 1.0 a.u.f.s. at 210 nm. Peptides are luteinizing hormone releasing factor (LRF) analogs synthesized in our laboratory. Primary structure of LRF is: *p*Glu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly-NH_2.

Peptides	*Retention time (sec)*	*Molecular weight*
[Δ^3-LPro–*p*ClDPhe–DTrp–Ser–Tyr βAla–Pro–Arg–N$^{\alpha}$$CH_3$Leu–DTrp]	1293	1349.5
H_2N-[Δ^3-LPro–*p*ClDPhe–DTrp–Ser–Tyr HO-βAla–Pro–Arg–N$^{\alpha}$$CH_3$Leu–DTrp]	1236	1367.5
[Δ^3-DPro–*p*ClDPhe–DTrp–Ser–Tyr βAla–Pro–Arg–N$^{\alpha}$MeLeu–DTrp]	1280	1349.5
H_2N-[Δ^3-DPro–*p*ClDPhe–DTrp–Ser–Tyr HO-βAla–Pro–Arg-N$^{\alpha}$MeLeu-DTrp]	1241	1367.5

CONCLUSION

The volatility of TEAF or the UV transparence and compatibility of TEAP with most biological systems make both solvent systems more versatile than those which contain detergents or high salt concentration. The molecular-weight range in which this system can be used (1000–44,000) is probably dependent on the original pore size of the derivatized silica. Derivatized silica with larger pore size or from other suppliers may allow for molecular-weight determinations greater than 44,000 using these particular buffer systems.

We have not investigated the detrimental effects of both low pH and high temperature on the integrity (chemical and biological) of the peptides and proteins used in these studies. It is certain that some proteins or enzymes will be sensitive to those denaturing conditions whereas others may not. The column support, on the other hand, may be used for long periods ($>$ 300 h of operation). Retention times, however, will vary significantly with age; compare, for example, data in Table I, column 2 with data (identical in terms of conditions presented in Table II which were run three months later after the column had experienced more than 100 operating h). Even though day-to-day repeatability is seemingly good, one should not expect laboratory-to-laboratory reproducibility since performance will be dependent on the condition of the columns.

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